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13. ABSTRACT *(Maximum 200 Words)*

Wnt signaling molecules have been implicated in mouse mammary carcinogenesis. β-catenin, a downstream molecule in the Wnt pathway, activates transcription of target genes. Our studies are aimed at determining whether β-catenin can function as an oncogene in the mammary gland. Using the mouse as a model system, we targeted expression of β-catenin to the mammary gland in transgenic animals. Our results show that transgenic females develop mammary gland hyperplasia, with the majority of them proceeding to develop mammary gland tumors. The β-catenin tumors are characterized as microacinar adenocarcinomas, nearly identical to those found in Wnt-induced mouse models. We were also interested in identifying downstream transcriptional targets of β-catenin in the mammary gland. We have found cyclin D1 and c-myc, targets previously identified in other systems, to be upregulated in β-catenin mammary tumors and tumor cell lines. In an attempt to identify novel targets of β-catenin, mammary cell lines expressing inducible or constitutively activated β-catenin were generated, and a whole genome screen appoach was taken. Several novel candidate target genes have been identified. Thus, our studies establish β-catenin as an oncogene in the mammary gland and have aided in the identification of gene targets in β-catenin-mediated mammary oncogenesis.

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Body

The Wnt signal transduction pathway has been shown to play a causative role in breast cancer in the mouse. β-catenin, which functions downstream in the Wnt signal transduction pathway, has been implicated in many types of human cancers. We were interested in determining whether β-catenin may play a role in mammary tumorigenesis. In an attempt to test whether β-catenin can function as an oncogene in the mammary gland, the mouse was chosen as a model system and a transgenic approach was taken.

An MMTV-promoter was used to target expression of a constitutively active form of β-catenin, lacking the 90 amino-terminal amino acids of the protein (ΔN), to the mammary gland. Four transgenic lines were generated from founder animals. Cohorts of continuously breeding females as well as virgin females from line ΔN-9281, expressing high levels of the transgene, as well as line ΔN-9801, expressing moderate levels of the transgene, were carefully monitored. Whole mount analysis of (tumor-free) mammary glands from virgin ΔN-9281 females revealed extensive regions of hyperplasia, with more limited areas of hyperplasia observed in mammary glands from ΔN-9801 virgin females. Moreover, mammary gland tumors were observed in line ΔN-9281 with roughly 40% of multiparous mice bearing tumors by 8 months of age, and nearly 70% developing tumors by 15 months. Multiparous females from the ΔN-9801 line also developed mammary

tumors, although the time course of tumor induction was somewhat delayed, consistent with the lower level of transgene expression in that line. Several of the mice were found to have multiple primary mammary tumors. Taken together, these results strongly support the notion that β-cateninΔN is sufficient to induce tumorigenesis in the mammary gland. Histopathologic analysis has demonstrated the majority of the tumors to fall into the category of microacinar adenocarcinomas. This pathology is nearly identical to that found in MMTV-Wnt-1 animals, supporting the notion that β-catenin is downstream of Wnt with respect to tumorigenicity. Cohorts of male animals from both the ΔN-9281 and ΔN-9801 transgenic lines were also followed, although there was no evidence of hyperplasia or tumorigenesis in the males. Results from the β-cateninΔN transgenic mice have been published (J. Michaelson and P. Leder, Oncogene, 2001).

Genetic collaboration in mammary oncogenesis, whereby two oncogenes together vastly accelerate the onset of tumorigenesis, has been observed in several instances. In particular, in mice bearing both MMTV-Wnt-1 and MMTV-Int-2 (Fgf3) transgenes, onset of tumors is significantly more rapid as compared to mice harboring only a single transgene. Given β-catenin's role in the Wnt pathway, we are interested in testing whether β-catenin can collaborate with Fgf3 in accelerating tumorigenesis. Double transgenics, carrying both constitutively active β-catenin and Fgf3 transgenes expressed in the mammary gland, were

generated and monitored for appearance of tumors. An accelerated tumorigenic phenotype has been observed in double transgenic animals, suggesting that the β-catenin pathway specifically collaborates with Fgf-3 in mammary gland tumorigenesis.

A second β-catenin trangenic mouse model has also been generated. An MMTV-promoter was used to drive expression of a carboxy-terminal truncated form of β-catenin (ΔC) to the mammary gland. The C-terminal truncation deleted the carboxy-terminal putative transactivation domain, thought to be required for activation of downstream targets. Three β-cateninΔC transgenic lines were generated, all of which express the transgene in the pregnant mammary gland. The highest expressing transgenic line, ΔC-3035 was chosen to be bred with MMTV-Wnt-1 transgenic mice. Double transgenic mice were generated and monitored for appearance of mammary tumors. The expectation was that Wnt-1 mediated mammary tumorigenesis might be halted or at least delayed due to the presence of a C-terminally deleted β-catenin molecule. Interestingly, however, double transgenics were found to have significantly *increased* rates of tumorigenesis, in contrast to what was expected. Further analysis is being carried out in the laboratory in an attempt to explain this interesting, yet unanticipated, finding.

In an attempt to enable further dissection of the molecular pathways involved in β-catenin-mediated mammary tumorigenesis, tumor cell lines were generated from the β-catenin-ΔN mammary tumors. Several tumor cell lines were generated, all of which expresses moderate to high levels of the transgene, as monitored by both Northern and/or Western blot analysis. In addition, tumor cell lines from MMTV-Wnt-1 mammary tumors were established. A panel of β-catenin and Wnt tumors and tumor cell lines were used to verify downstream transcriptional targets of β-catenin in the transformed mammary gland. Indeed, c-myc and cyclin-D1, previously identified β-catenin targets in other cellular contexts, were found to be upregulated in the panel of β-catenin and Wnt mammary tumors and tumor cell lines as assessed by Northern blot analysis. (J. Michaelson and P. Leder, Oncogene, 2001).

To aid in the identification of novel downstream targets of β-catenin, an inducible expression system for β-catenin in a mammary epithelial cell line was established. Both β-cateninΔN and β-catenin-ΔC were placed under a promoter that requires tTA for expression. These constructs were introduced by stable transfection into a clone of the mammary epithelial cell line Eph4 expressing tTA in a tet-inducible fashion. Several clones expressing either inducible β-catenin-ΔN or β-catenin-ΔC were identified. The β-catenin inducible expressing cell lines were used in a genome-wide screen to identify target genes that are up- or down-

regulated in response to induced β-catenin expression. Approximately thirty novel putative targets of β-catenin were identified. Follow up studies to verify these targets is being pursued in the laboratory. To aid in target verification, several clones stably expressing high levels of β-catenin have also been generated. Ultimately, the β-catenin-derived tumor cell lines will be used as an additional means to verify targets and will further provide the opportunity to demonstrate the relevance of these targets to tumorigenesis *in vivo*.

The studies outlined here provide convincing evidence that β-catenin plays a causative role in mammary gland tumorigenesis. Furthermore, these results support the notion that the tumorigenicity associated with the Wnt signal transduction pathway is mediated by β-catenin. These studies also demonstrate that c-myc and cyclin D1 are likely targets of β-catenin in the mammary gland, and have also provided the basis for identification of novel targets of β-catenin in the mammary gland. Taken together, this work has helped to elucidate the role of β-catenin in mammary gland tumorigenesis.

Key Research Accomplishments

- Established MMTV-driven constitutively N-terminally truncated activated β-catenin transgenic mouse lines

- Found tumors (predominantly microacinar adenocarcinoma) in mice expressing β-catenin transgene

- Found accelerated rates of mammary gland tumorigenesis in mice expressing both activated β-catenin and Fgf-3 transgenes

- Established MMTV-driven C-terminally truncated β-catenin transgenic mouse lines

- Found accelerated rates of mammary gland tumorigenesis in mice expressing both C-terminally truncated β-catenin and Wnt-1 transgenes

- Established mammary tumor cell lines derived from β-catenin murine mammary tumors

- Established mammary cell lines with inducible expression of β-catenin

- Established mammary cell lines constitutively overexpressing β-catenin

- Identified ~30 novel potential targets of β-catenin

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Reportable Outcomes

Manuscript:

Michaelson, J.S. and Leder, P. Oncogene **20**: 5093 (2001).

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Mouse Models:

Transgenic: MMTV-driven constitutively N-terminally truncated activated β-catenin transgenic mouse lines (MMTV-βcatenin ΔN)

Transgenic: MMTV-driven C-terminally truncated β-catenin transgenic mouse lines (MMTV-βcatenin ΔC)

Bi-transgenic: MMTV-βcatenin ΔN *AND* MMTV-Fgf3

Bi-transgenic: MMTV-βcatenin ΔC *AND* MMTV-Wnt-1

Cell Lines:

Mammary tumor cell lines derived from MMTV-β-cateninΔN murine mammary tumors

Mammary tumor cell lines derived from MMTV-Wnt-1 murine mammary tumors

Mammary cell lines with inducible expression of β-catenin

Mammary cell lines constitutively overexpressing β-catenin

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Conclusions

Our studies demonstrate that β-catenin can function as an oncogene in inducing tumorigenesis in the mouse mammary gland. Transgenic animals expressing activated β-catenin in the mammary gland, which were generated in the course of these studies, developed hyperplasia and eventually tumors in the mammary gland. We found that the histopathology of the tumors was nearly identical to that found in Wnt induced mammary tumors, confirming the notion that β-catenin mediates Wnt-induced tumorigenesis.

Our studies have also aided in the identification of downstream target genes of β-catenin in the mammary gland. We have demonstrated that cyclin D1 and c-myc, known targets of β-catenin in other cellular contexts, are also upregulated in β-catenin tumors and tumor cell lines.

Another aspect of our studies has addressed the issue of genetic collaboration in mammary oncogenesis. We have found that bi-transgenic animals expressing both Fgf-3 and activated β-catenin in the mammary gland have an accelerated rate of tumorigenesis as compared to single transgenic animals. These findings, together with previous studies showing collaboration between Fgf-3 and Wnt-1, suggest that β-catenin is sufficient to mediate the collaboration observed in the case of Wnt-1.

Recently, it was shown that upregulated levels of β-catenin have been found in human breast tumors. Our studies are thus of particular significance since they demonstrate a causative role for β-catenin in breast cancer. In addition, our work has aided in dissecting the genetic and biochemical pathways responsible for the oncogenic nature of β-catenin.

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β-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland

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The Wnt signal transduction pathway has been implicated in mammary tumorigenesis in the mouse. β-catenin, a key downstream effector of this pathway interacts with and thus activates the Tcf/Lef family of transcription factors. Elevated levels of β-catenin have been found in many human tumors, notably colon carcinomas. Recently, elevated levels of β-catenin have been associated with poor prognosis in human adenocarcinoma of the breast. In order to assess the possible role of β-catenin in mammary carcinoma, we have created transgenic mice bearing the MMTV–LTR driving an activated form of β-catenin. These mice develop mammary gland hyperplasia and mammary adenocarcinoma, a phenotype very similar to that of transgenic mice expressing an MMTV-driven Wnt gene. Indeed, the histopathology of the mammary tumors in Wnt-mediated adenocarcinoma is identical to that observed in our β-catenin-mediated disease model. Furthermore, putative β-catenin transcriptional targets, cyclin D1 and c-*myc*, are elevated in β-catenin-mediated mammary tumors and cell lines. These observations support the notion that the oncogenic Wnt pathway operates via β-catenin and its targets in the context of mammary hyperplasia and carcinoma. *Oncogene* (2001) **20,** 5093–5099.

Keywords: β-catenin; breast cancer; mammary gland; Wnt; adenocarcinoma; MMTV

Introduction

β-catenin has a diverse set of functions within the cell. In its capacity to bind components of the actin cytoskeleton, namely E-cadherin and α-catenin, β-catenin plays a role in cell adhesion (reviewed in Bullions and Levine (1998)). β-catenin is also an integral player in the Wnt signal transduction pathway (reviewed in Polakis (1999, 2000)). In a resting cell, β-catenin is phosphorylated by glycogen synthase kinase-3β (GSK) in association with adenomatous polyposis coli (APC) protein, an event which targets β-catenin for ubiquitination and subsequent degradation. In response to extracellular Wnt, a signal is transduced through the membrane-bound Frizzled receptor and subsequently through Disheveled, facilitating inhibition of GSK3β. The net result of the Wnt signal is cytoplasmic accumulation of β-catenin and entry of β-catenin into the nucleus. Once in the nucleus, β-catenin associates with Tcf/Lef transcription factors and together these activate downstream targets. Down-regulation of β-catenin levels in the nucleus likely occurs via nuclear export by APC, as recently reported (Henderson, 2000; Neufeld *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2000).

Structure-function analyses of β-catenin and its *Drosophila* homolog, Armadillo, have been informative. Amino-terminal deletion mutants were shown to have increased stability due to loss of GSK phosphorylation residues (Barth *et al.*, 1997; Munemitsu *et al.*, 1996). The central portion of β-catenin, comprised of 12 armadillo repeats, facilitates multiple protein interactions, including binding to Tcf/Lef, APC, E-cadherin and α-catenin (Orsulic and Peifer, 1996; van de Wetering *et al.*, 1997). The carboxy-terminus functions as a transcriptional activation domain, as demonstrated in GAL4 transcriptional assays (van de Wetering *et al.*, 1997). An additional, albeit somewhat weaker, transactivation domain may reside in the N-terminal portion of the protein (Hsu *et al.*, 1998).

A correlation between β-catenin and tumorigenesis has been well established (reviewed in Polakis (2000)). Mutations and deletions of phosphorylation sites in the amino terminus resulting in stabilization of β-catenin have been identified in numerous human tumors and cancer cell lines, including colon, hepatocellular, ovarian, endometrial and others (reviewed in Polakis (2000)). Elevated β-catenin levels resulting from mutations in other components of the Wnt signaling pathway, such as loss-of-function APC mutations, are also commonly found. In colon cancer, it is estimated that nearly 85% of tumors contain mutations in APC, with a majority of the remainder containing activating β-catenin mutations (Kinzler and Vogelstein, 1996; Morin *et al.*, 1997). Mouse models of β-catenin overexpression have recently been generated in an attempt to show a causative role for β-catenin in the onset of tumorigenesis. Gat *et al.* (1998) demonstrated that mice expressing K14-driven β-catenin (ΔN87) develop benign hair follicle tumors. In contrast, when placed under the control of the intestinal *Fabp1* promoter, β-catenin (ΔN89) mice did not develop

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intestinal polyps, although increased branching of the intestinal villi was observed (Wong *et al.*, 1998). In a subsequent study, intestinal polyps were observed when β-catenin (Δex3) was introduced into the endogenous β-catenin locus by homologous recombination (Harada *et al.*, 1999).

The mammalian transcriptional targets of β-catenin are beginning to be elucidated. Using SAGE analysis, c-*myc* was identified as a target of the pathway in human colorectal cells (He *et al.*, 1998). A search of the database for canonical Tcf/Lef binding sites identified cyclin D1 as an additional β-catenin target (Tetsu and McCormick, 1999). That study, as well as those of Shtutman *et al.* (1999) demonstrated regulation of cyclin D1 by β-catenin in colon carcinoma cell lines. However, it is likely that at least some β-catenin targets may be restricted to specific cell types. For example, c-*myc* was not uniformly activated in RK3E cell lines (E1A-immortalized epithelial cell line derived from neonatal rat kidney) transformed by β-catenin (Kolligs *et al.*, 1999).

Components of the Wnt signaling pathway have been definitively linked to mammary tumorigenesis. Several Wnt family members can induce morphological transformation and altered growth characteristics in cultured C57MG mammary epithelial cells (Brown *et al.*, 1986; Wong *et al.*, 1994). Moreover, studies from our laboratory and others using MMTV transgenic mouse models have demonstrated that both Wnt-1 and Wnt-10b are capable of inducing mammary adenocarcinomas (Lane and Leder, 1997; Tsukamoto *et al.*, 1988). Mammary tumors were also observed in heterozygous APC^{Min} mice (Moser *et al.*, 1993). Regarding β-catenin, a recent study indicates that β-catenin activity in human breast tumors correlates with elevated expression of cyclin D1 as well as a poor prognosis (Lin *et al.*, 2000). A causative role for β-catenin in mammary tumorigenesis, however, has not been established.

In this study, we explore directly whether β-catenin can induce mammary tumorigenesis and, thus, could serve as a downstream effector of the Wnt pathway. To that end, we have generated transgenic mice expressing an activated form of β-catenin in the mammary gland (MMTV-βcatΔN). We find that MMTV-βcatΔN mice, like mice bearing a Wnt transgene, exhibit hyperplasia of the mammary gland. Furthermore, MMTV-βcatΔN mice develop mammary adenocarcinomas, with a histopathology that is identical to that found in MMTV-Wnt transgenic mice. Finally, we show that putative mammalian targets of β-catenin are elevated in MMTV-βcatΔN tumors and tumor cell lines.

Results

MMTV-βcatΔN transgenic mice

Transgenic mice were generated to investigate a causative role for β-catenin in mammary gland oncogenesis. The transgenic construct consists of an MMTV promoter placed upstream of a truncated β-catenin cDNA lacking the first 90 codons (β-cateninΔN90) tagged with a 3′-KT3 epitope followed by an SV40 polyadenylation (polyA) site. Following injection of the construct into the male pronucleus of embryos and transfer to pseudopregnant foster mothers, 44 pups were born. From these, four transgenic founder mice were identified by Southern blot analysis using a probe for SV40 polyA. All four founder mice passed the transgene to progeny, thereby generating four independent transgenic lines, referred to as MMTV-βcatΔN.A-D. Expression analysis revealed that the transgene was expressed in three out of four transgenic lines, namely MMTV-βcatΔN.A–C. The MMTV-βcatΔN.A and MMTV-βcatΔN.B lines were expanded and chosen for further analysis.

Transgene expression in both MMTV-βcatΔN.A and MMTV-βcatΔN.B lines is shown in Figure 1. Northern blot analysis using an SV40 polyA probe detects a high level of expression of the transgene in mammary glands isolated from day 17.5 pregnant females from MMTV-βcatΔN.A and moderate expression levels in MMTV-βcatΔN.B (Figure 1a). To confirm expression at the protein level, Western blot analysis of mammary gland lysates prepared from day 17.5 pregnant MMTV-βcatΔN.A mice was performed. An antibody to β-catenin detects endogenous β-catenin in all samples as well as the truncated β-cateninΔN90 protein in transgenic mammary glands from MMTV-βcatΔN.A, but not from wildtype FVB or MMTV-βcatΔN.D (non-expressing transgenic) mammary glands (Figure 1b). Analysis of transgene expression in a range of tissues derived from MMTV-βcatΔN.A mice reveals the highest levels of expression in the mammary gland, with low levels of expression in a limited number of other tissues, including testis and skeletal muscle (Figure 1c). In MMTV-βcatΔN.B, transgene expression is detected exclusively in the mammary gland (Figure 1d), albeit at lower levels than in MMTV-βcatΔN.A. Thus, the MMTV promoter reliably directs expression of the β-cateninΔN90 transgene to the mammary gland in MMTV-βcatΔN mice.

Mammary gland hyperplasia in MMTV-βcatΔN transgenic mice

Whole mount analysis was performed to examine the ductal structure of mammary glands in MMTV-βcatΔN transgenic mice. As shown in Figure 2, examination of a mammary gland taken from a wildtype FVB female mouse reveals a branching ductal structure typical of a virgin female. In contrast, inspection of an MMTV-βcatΔN.A mammary gland reveals a mosaic pattern characterized by normal ductal structures interspersed with patches of dense epithelium. Examination of a mammary gland from an MMTV-βcatΔN.B virgin female similarly reveals a mosaic pattern, with areas of dense epithelium represented to a lesser extent, consistent with the lower level of expression of the transgene in that line. The areas of dense epithelium detected in the transgenic

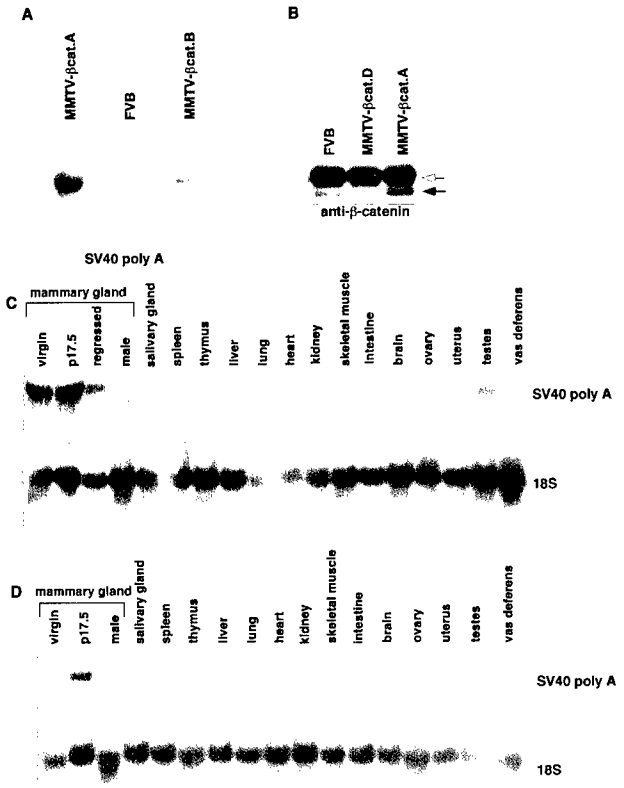

Figure 1 (**a**) MMTV-βcatΔN directs expression of activated β-catenin to the mammary gland. Northern blot analysis of RNA isolated from mammary glands of 17.5 day pregnant transgenic or wildtype FVB females. Probe detects SV40 poly A tail present in transgenic transcript. (**b**) Western blot analysis of protein lysate prepared from mammary glands of 17.5 day pregnant transgenic or wildtype FVB females. Anti-β-catenin antibody detects endogenous β-catenin protein in all samples (indicated by open arrow) as well as β-cateninΔN90 truncated protein (indicated by closed arrow) present in lysates from transgene expressing line (MMTV-βcatΔN.A) only and migrating just above a faint non-specific band. Northern blot of RNA prepared from tissues isolated from (**c**) MMTV-βcatΔN.A and (**d**) MMTV-βcatΔN.B transgenic animals. p17.5 = 17.5 day pregnant female mammary gland

mammary glands are indicative of mammary gland hyperplasia. The presence of hyperplastic mammary tissue has been confirmed by hematoxylin and eosin staining of transgenic mammary gland sections (data not shown). Significantly, these findings indicate that overexpression of an activated form of β-catenin in the mouse mammary gland is sufficient to induce hyperplasia.

MMTV-βcatΔN transgenic mice develop mammary adenocarcinomas

Activated β-catenin cannot only induce hyperplasia of the mammary gland, but is also capable of resulting in neoplasia. A cohort of 16 MMTV-βcatΔN.A continuously breeding female mice was followed and inspected on a weekly basis for mammary tumors for a period of 15 months. As shown in Figure 3a, MMTV-βcatΔN.A mice develop mammary tumors at a relatively rapid rate, with roughly 40% of mice bearing tumors by 8 months of age, and nearly 70% developing tumors by 15 months. In many of these animals, multiple independent tumors arise. Multiparous MMTV-βcatΔN.B females also develop mammary tumors, although the time course of tumor induction is somewhat delayed, consistent with the lower level of transgene expression in that line. Despite the dependence of the MMTV promoter on successive rounds of pregnancy and lactation for high level expression, several virgin females also developed mammary tumors by 12 months of age (3/14 in MMTV-βcatΔN.A (Figure 3a); 3/14 in MMTV-βcatΔN.B). The founder female from the third transgenic line, MMTV-βcatΔN.C, also developed a mammary tumor, although this line was not further expanded. The appearance of mammary tumors in three independent transgenic lines strongly supports MMTV-βcatΔN as sufficient to induce tumorigenesis in the mammary gland.

The histopathology of MMTV-βcatΔN tumors was assessed by examination of hematoxylin and eosin stained tumor sections (Figure 3b). Tumors arising from all three MMTV-βcatΔN lines are consistent with classification as microacinar adenocarcinomas. The tumors are characterized by highly disorganized tissue with patches of cells forming small acini lined by neoplastic cells. Under high magnification, it is evident that the cells have pleiomorphic nuclei with prominent nucleoli, that the cytoplasm is sparse with a large number of vacuoles, and that there is a high mitotic index among the tumor cells (data not shown). In some cases, invasion into neighboring normal mammary gland tissue is apparent. There is no evidence of metastases to other organs sites. Significantly, the histopathology of the MMTV-βcatΔN tumors is identical to that observed in MMTV-Wnt tumors (Lane and Leder, 1997; Tsukamoto *et al.*, 1988), supporting an oncogenic role of β-catenin in the Wnt pathway in the mammary gland.

Activation of β-catenin downstream target genes in MMTV-βcatΔN tumors and cell lines

The ability of β-catenin to induce neoplasia in the mammary gland is likely a result of transcriptional activation of downstream target genes. Candidate target genes have been identified, e.g. cyclin D1 and c-*myc*, in colorectal cells, although it is unclear whether these are also targets in other organ systems. Our β-catenin tumors provide an *in vivo* model to test for target gene upregulation in the mammary gland. RNA prepared from a panel of tumors derived from MMTV-βcatΔN and MMTV-Wnt animals was subjected to Northern blot analysis. Both cyclin D1 and c-*myc* are upregulated in the majority of β-catenin tumors (11/12), particularly in those expressing high levels of the transgene, and in all (3/3) Wnt-1 tumor cell lines (Figure 4).

Cell lines derived from tumors provide a homogenous population of epithelial cells and thus an additional model in which to test for target gene

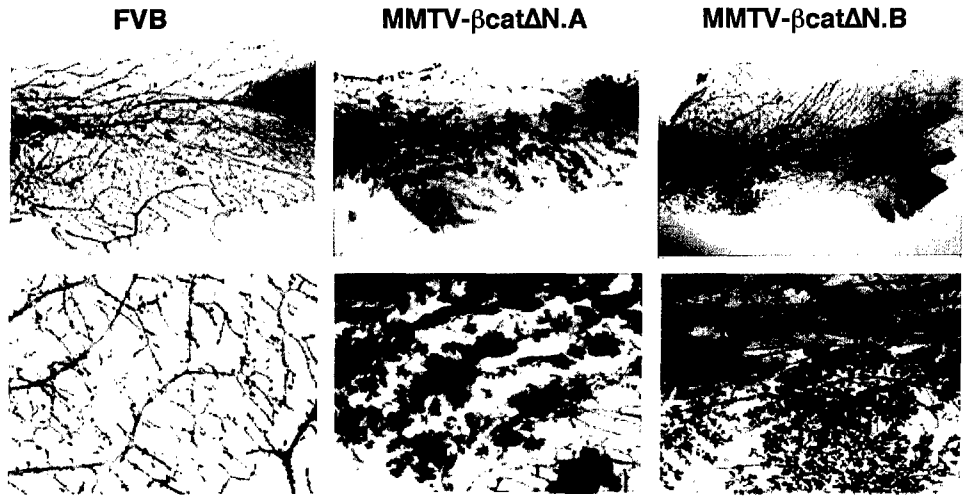

Figure 2 Whole mount analysis of transgenic mammary glands reveals hyperplasia. Whole mount analysis of mammary glands from 1 year old virgin transgenic or wildtype FVB females. Lower panels show high magnification of selection from upper panel

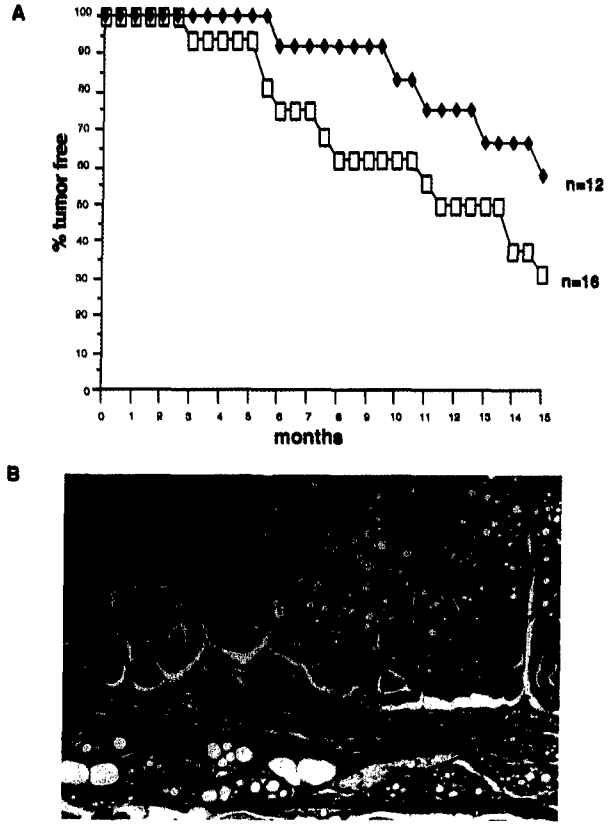

Figure 3 MMTV-βcatΔN develop mammary gland microacinar adenocarcinomas. (**a**) Survival curve of cohort of multiparous (open rectangles) and virgin (closed diamonds) MMTV-βcatΔN.A mice. (**b**) Hematoxylin and eosin staining of tumor section from MMTV-βcatΔN tumor

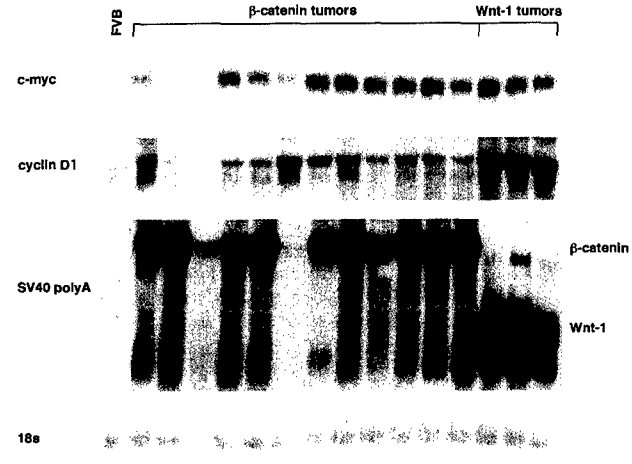

Figure 4 Transcriptional targets are upregulated in β-catenin and Wnt-1 tumors. Northern blot analysis of RNA isolated from wildtype FVB virgin mammary glands, β-catenin mammary tumors, or Wnt-1 mammary tumors

expression. Ten independent epithelial cell lines were established from five original MMTV-βcatΔN tumors following 6–10 months in culture. Transgene expression is dexamethasone dependent, characteristic of the glucocorticoid-sensitive MMTV–LTR, as shown in representative tumor cell lines (Figure 5a). Expression at the protein level of the β-catenin-ΔN90 was detected by Western blot with either an antibody against β-catenin or an antibody against the KT3 epitope tag (Figure 5b).

To test whether the β-catenin-ΔN90 protein is transcriptionally active in the tumor cell lines, a luciferase reporter assay was employed. Tumor cell lines were transfected with a pTOPFLASH reporter construct, consisting of multimerized Tcf/Lef binding sites upstream of a luciferase reporter gene (van de Wetering *et al.*, 1997), and assayed for luciferase activity. Dexamethasone-inducible transcriptional activity is detected in the tumor cell lines but not in a normal mammary epithelial cell line, Eph4 (Figure 5c).

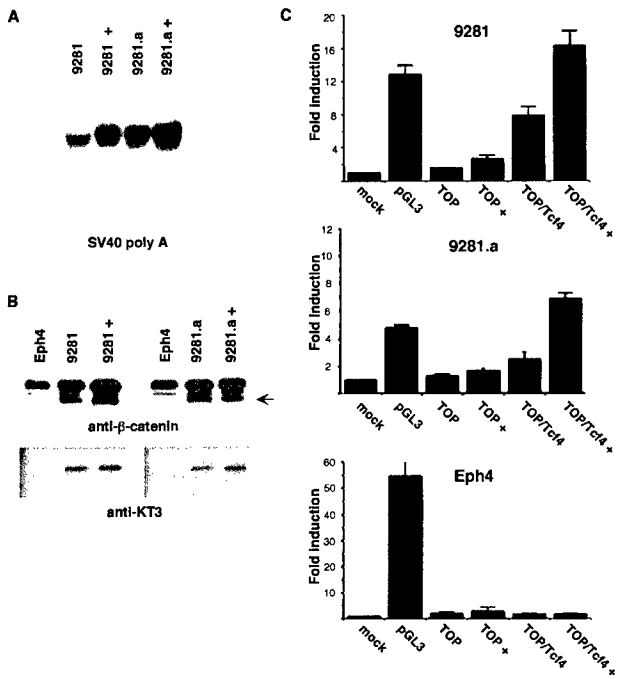

Figure 5 β-cateninΔN90 is expressed and transcriptionally active in MMTV-βcatΔN tumor cell lines. (**a**) Northern blot analysis of RNA isolated from two independently derived MMTV-βcatΔN tumor cell lines, 9281 and 9281.a. Cells grown in the presence of dexamethasone are marked with a plus sign. (**b**) Western blot analysis of lysates prepared from MMTV-βcatΔN tumor cell lines or a normal epithelial cell line, Eph4. Anti-β-catenin antibody detects endogenous β-catenin as well as β-cateninΔN90 (indicated by arrow). Anti-KT3 antibody detects the tagged β-cateninΔN90. (**c**) Luciferase assays following transient transfection into tumor cell lines or Eph4 control cell line. TOP refers to pTOPFLASH Tcf reporter construct. Dexamethasone treated cells are marked with a plus sign. y-axis shows fold-induction over mock-transfected sample. Values shown are average of three independent transfections with standard deviation indicated as bars

Increased levels of transcriptional activity are detected in the tumor cell lines upon cotransfection with a Tcf-4 cDNA construct, likewise in a dexamethasone-inducible fashion. These studies establish that the β-catenin-ΔN90 protein is expressed in MMTV-β-catΔN tumor cell lines and, furthermore, is capable of activating transcription of downstream targets.

Target gene expression was assessed in the β-catenin tumor cell lines. Both cyclin D1 and c-*myc* are upregulated in all of the tumor lines (10/10) (Figure 6). Taken together, our data suggest that cyclin D1 and c-*myc* are likely to be *in vivo* targets of β-catenin in the mammary gland.

Discussion

We have generated a mouse model in which an activated form of β-catenin (MMTV-βcatΔN) is overexpressed in the mammary gland. Our findings provide a definitive demonstration that β-catenin can induce mammary tumorigenesis. Previously, overexpression of upstream components of the Wnt signal transduction pathway, namely Wnt-1 and Wnt-10b were shown to be oncogenic in the mouse mammary gland. The histopathology of the MMTV-βcatΔN tumors is identical to that observed in tumors derived from MMTV-Wnt-1 and MMTV-Wnt-10b. Together, our observations support the notion that β-catenin is likely responsible for the oncogenicity previously associated with Wnt molecules in the mammary gland.

Interestingly, we have observed that MMTV-βcatΔN male mice do not exhibit a mammary gland phenotype. This is in contrast to MMTV-Wnt males which develop ductal formations in the mammary fat pad, particularly striking in the case of Wnt 10b (Lane and Leder, 1997). Both Wnt-1 and Wnt-10b males further proceed to develop mammary adenocarcinomas, albeit at a relatively low frequency (Lane and Leder, 1997; Tsukamoto *et al.*, 1988). In contrast, whole mount analysis revealed only rudimentary epithelial buds in MMTV-βcatΔN males, similar to that found in wild-type males (data not shown). Moreover, none of the MMTV-βcatΔN male mice (MMTV-βcatΔN.A, 0/13; MMTV-βcatΔN.B, 0/6) developed mammary adenocarcinomas by 12 months of age (data not shown). This disparity may be due to differing levels of transgene expression in the male, although the transgene is expressed at significant levels in MMTV-βcatΔN.A males (Figure 1c). The possibility that MMTV-βcatΔN males might eventually develop tumors at a later age also cannot be ruled out. Of note, tumor development in females also has a longer latency in MMTV-βcatΔN mice as compared to those in the MMTV-Wnt models. Intriguingly, the difference could potentially be attributed to β-catenin-independent Wnt signaling (Haertel-Wiesmann *et al.*, 2000; Ziemer *et al.*, 2001) which may activate pathways the consequence of which is an acceleration of the rate of oncogenesis. The role of alternate downstream components of the Wnt pathway in mammary tumorigenesis therefore cannot be ruled out.

The MMTV-βcatΔN female mice exhibit mammary gland hyperplasia. In the *Fabpl*-β-catenin (ΔN87)

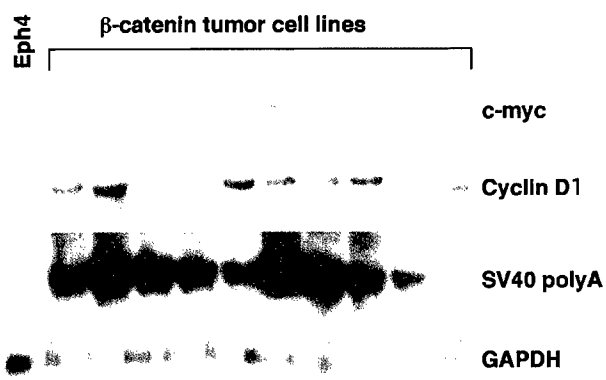

Figure 6 Transcriptional targets are upregulated in β-catenin tumor cell lines. Northern blot analysis of RNA isolated from a normal mammary epithelial cell line, Eph4, and from β-catenin mammary tumor cell lines

mouse model, of particular interest given the importance of the APC/β-catenin pathway in colon cancer, animals exhibit hyperplasia of the intestinal villi (Wong *et al.*, 1998). However, these mice do not develop intestinal adenocarcinomas. In a more recent report on β-catenin in the intestine, polyp formation was observed when an activated form of β-catenin was expressed from the endogenous locus in intestinal epithelial cells (Harada *et al.*, 1999). In the keratin promoter model (Gat *et al.*, 1998), in addition to de novo hair follicle growth, two types of benign tumors were observed in the animals expressing activated β-catenin. In our mammary gland model, we have found that a significant percentage of mice expressing activated β-catenin develop adenocarcinoma of the breast. The strength of the phenotype observed in the MMTV-βcatΔN mice is suggestive of the potency of β-catenin as an oncogene in the mammary gland.

β-catenin likely induces tumorigenesis through its ability to transactivate downstream target genes. It is consistent, therefore, that cyclin-D1 and c-*myc*, known oncogenes are targets of β-catenin. We have found that these target genes are upregulated in the vast majority of β-catenin mammary tumors and tumor cell lines, suggesting that they are *in vivo* targets of β-catenin in the mammary gland. We have also observed Wisp-1 and Wisp-2 (Wnt induced secreted proteins), genes identified as Wnt/β-catenin targets in Wnt-1 transformed C57MG mammary epithelial cells (Pennica *et al.*, 1998; Xu *et al.*, 2000), as being upregulated in β-catenin tumor cell lines (data not shown). However, the possibility that the elevated levels of target genes that we have observed are a result of secondary hits occurring during progression of the tumorigenic process or establishment of the cell lines in culture cannot be ruled out.

The MMTV-βcatΔN construct used to generate the transgenic mice lacks the N-terminal GSK3β phosphorylation sites yet retains the C-terminal activation domain of β-catenin. Interestingly, Hsu *et al.* (1998) showed that the N-terminal portion of β-catenin, which is deleted in our transgenic construct, also possesses transcriptional activity. Our findings suggest that the C-terminal transcriptional domain is sufficient for oncogenesis *in vivo* in the mammary gland. Furthermore, the transcriptional targets that we have identified as upregulated in MMTV-βcatΔN tumors are necessarily activated independent of the N-terminal activation domain of β-catenin.

Elevated levels of β-catenin were recently observed in a subset of human breast cancers and a correlation was made with a poor prognosis in that patient subgroup (Lin *et al.*, 2000). In human studies, it is difficult to determine whether β-catenin plays a causative role in tumor formation or whether its upregulation is a secondary effect of the tumorigenic process. Our studies demonstrating the oncogenic capacity of β-catenin in the mouse mammary gland support the notion that activating mutations of β-catenin mutations may represent primary events in the development of human breast cancer.

Materials and methods

Transgenic mice

To generate the MMTV-βcatΔN90 construct, the *Sac*II/*Xba*I fragment from ΔN90 mouse β-catenin cDNA (a kind gift from W Nelson, Stanford University; Barth *et al.*, 1997) was cloned into the *Sac*II and *Xba*I sites of pSL301 (Invitrogen), and subsequently removed from pSL301 by digestion with *Xba*I and *Xho*I, followed by *n*-fill with Klenow enzyme. The ΔN90 β-catenin fragment was then blunt-end ligated into the *Eco*RI site (*n*-filled) of the MMTV-SV40-Bssk vector, between the murine mammary tumor virus long terminal repeat (MMTV−LTR) and an SV40 large T antigen intron and polyadenylation signal. Prior to injection, the construct was linearized with *Sal*I and gel purified.

Five nanograms of DNA was injected into the male pronucleus of one-cell stage FVB/N inbred mouse embryos. Pseudo-pregnant SW foster mothers were reimplanted with viable embryos. Tail DNAs isolated from offspring were tested for the presence of the transgene by enzyme restriction with *Bam*HI followed by Southern blot analysis and probing with a 0.9 kb *Hind*III/*Bam*HI SV40 poly A fragment.

Northern blot analysis

RNA was isolated from tissues, tumors and cell lines using RNA STAT-60 (Tel-Test). For RNA blot analysis, 15 ug of total RNA was heat denatured and separated on a 1% agarose gel containing formaldehyde and MOPS. RNA was transferred to a nylon GeneScreen membrane (NEN) in 10×SSC and then fixed to the membrane with UV light. Hybridization was carried out in buffer containing 50% formamide/50 mM sodium phosphate/0.8 M NaCl/10 mM EDTA/2.5×Denhardts/0.2% SDS/0.34 mg/ml yeast RNA (Gibco-BRL)/0.4 mg/ml sonicated herring sperm DNA.

Transgene expression was detected with the 0.9 kb *Hind*III/*Bam*HI SV40 poly A fragment. The c-*myc* probe is 5.6 kb and spans exons 2 and 3. A probe for cyclin D1 was generated by RT−PCR with oligonucleotide primers corresponding to bp 608−630 and 968−989 of cyclin D1 cDNA (Smith *et al.*, 1995). A probe for 18s rRNA was PCR generated with primers corresponding to bp 1244−1264 and 1645−1667 (GenBank accession # X00686.1).

Protein analysis

Mammary tissue or tumors were homogenized and lysates were prepared in buffer containing 20 mM HEPES/150 mM NaCl/1% Triton X-100/2 mM EDTA/2 mM EGTA with protease inhibitors. Lysates were run on 8% polyacrylamide gels and then transferred to a PVDF Immobulin-P membrane (Millipore). Monoclonal anti-β-catenin (Transduction Laboratories) and anti-KT3 (Babco) were used as primary antibodies. Horseradish peroxidase linked sheep anti-mouse Ig (Amersham) was used as a secondary antibody, followed by detection by ECL.

Histological analysis

To prepare whole mounts, mammary glands (# 4) were dissected, immediately placed on glass slides, and allowed to dry for 2−3 min. Fixation was carried out overnight at room temperature in Carnoy's solution (95% ethanol: chloroform: glacial acetic acid [6:3:1]). Following a rinse in 70% ethanol, samples were stained in carmine alum solution (0.2% carmine (Sigma) and 0.5% aluminum potassium sulfate (Sigma)) at

4 C overnight. Dehydration was performed in ascending concentrations of ethanol followed by clearing in xylenes. Whole mount samples were stored in methyl salicylate.

Samples were prepared for histologic analysis by placing fresh tissue in Optimal Fix (American Histological Reagent Co.). Paraffin embedded sections were analysed by Dr Robert Cardiff (Center for Comparative Medicine, University of California, Davis, USA).

Tumor cell culture

Tumor tissue was rinsed in PBS, minced with a surgical blade no. 21 (Bard-Parker), and then placed in culture in DMEM-F12 media (Gibco-BRL) containing 2.5% fetal calf serum (Sigma), 10 μg/ml insulin (Sigma), 5 ng/ml epidermal growth factor (Sigma) in the presence of antibiotics at 37°C with 7.5% CO_2. Fibroblasts were periodically removed from expanding tumor epithelial cell lines by brief trypsinization. Cell lines were established following an average of 4–6 passages and 6–10 months in culture. Dexamethasone (Sigma) treated cells were treated at 1 μM for 24 h.

Transient transfection and luciferase assays

The mammary epithelial cell line Eph4 was maintained in culture in DMEM media (Gibco-BRL) containing 10% bovine serum, 2% 200 mM L-glutamine and antibiotics at 37°C with 7.5% CO_2. Cells were grown to 40–50% confluency in 6-well plates and transiently transfected using Fugene6 reagent (Boehringer Mannheim) according to the protocol. Lysates were prepared 40–42 h following transfection in Passive Lysis Buffer (Promega) and analysed on an Automat LB953 luminometer (Berthold) with automatic injection of luciferase reagent (Promega). Luciferase assays were performed in triplicate. pTOPFLASH luciferase construct, containing three multimerized Tcf binding sites, and a *myc* tagged version of human Tcf-4, were kindly donated by H Clevers (van de Wetering *et al.*, 1997). pGL3 control vector (Promega), containing an SV40 promoter and enhancer, was used as a positive control in the luciferase assays.

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